K-*ras* and p53 in Pancreatic Cancer: Association with Medical History, Histopathology, and Environmental Exposures in a Population-based Study[1]

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Abstract

Pancreatic cancer is a highly fatal cancer with few identified risk factors. Increased risk of pancreatic cancer in tobacco smokers and among diabetic patients is well established, and some reports have suggested associations with coffee consumption and occupational exposure to organochlorines. At present, there is little information regarding the possible association of these risk factors with the known genetic alterations found in pancreatic cancers, such as activation of the K-*ras* oncogene and inactivation of the *p53* tumor suppressor gene. Knowledge of such relationships may help to understand the molecular pathways of pancreatic tumorigenesis. We investigated the association between these molecular defects and risk factors for pancreatic cancer in 61 newly diagnosed patients identified through an ongoing study of pancreatic cancer in the San Francisco Bay Area. Interview information was obtained regarding environmental exposures, medical history, and demographic factors. Serum levels of dichlorodiphenyltrichloroethylene (DDE) and polychlorinated biphenyls were available on a subset of 24 patients. Tumor blocks were located from local hospitals and used for K-*ras* mutational analysis at codon 12 and for p53 protein immunohistochemistry. The molecular analyses were facilitated through the use of laser capture microdissection, which provides a reliable method to obtain almost pure populations of tumor cells.

Mutations in K-*ras* codon 12 were found in 46 (75%) of 61 pancreatic cancers. A prior diagnosis of diabetes was significantly associated with K-*ras* negative tumors ($P = 0.002$, Fisher's exact test). The absence of this mutation was also associated with increased serum levels of DDE, although this association was not statistically significant ($P = 0.16$, Wilcoxon's test). There was no difference in polychlorinated biphenyl levels between the K-*ras* wild-type and mutant groups. Immunohistochemical staining for p53 protein did not differ by patient characteristics or clinical history, but significant associations were found with poor glandular differentiation ($P = 0.002$, χ^2 trend test), severe nuclear atypia ($P = 0.0007$, χ^2 trend test), and high tumor grade ($P = 0.004$, χ^2 trend test). Our results are suggestive of the presence of K-*ras* codon 12 mutation-independent tumorigenesis pathways in patients with prior diabetes and possibly in patients with higher serum levels of DDE. Our results also support a role for the p53 tumor suppressor protein in the maintenance of genomic integrity.

Introduction

Pancreatic carcinoma is the fifth leading cause of cancer death in the United States (1). This is mainly attributable to the extremely poor survival: less than 20% of newly diagnosed patients survive the 1st year, whereas the 5-year survival rate for all stages is less than 5%. Despite this poor outcome, considerable progress has been made in our understanding of the biology of pancreas carcinoma, with diagnosis and staging, and with treatment and palliation of the disease.

The causes of pancreatic cancer remain unknown, but there are several factors that increase the risk for the development of the disease (for reviews, see Refs. 2–4). Two risk factors have been firmly established: tobacco smoking and diabetes mellitus. Between one-fourth and one-half of the pancreas cancer cases can be attributed to cigarette smoking; several cohort and case-control studies have found an increased risk of 2- to 3-fold (5, 6). A meta-analysis of 20 case-control and cohort studies demonstrated that a history of diabetes mellitus preceding the diagnosis of pancreatic cancer by more than 1 year is associated with a 2-fold increased risk for pancreatic cancer (4). This risk factor had not been appreciated until recently, mainly because diabetes can be one of the symptoms associated with pancreatic cancer.

Dietary and environmental factors are suspected to influence pancreatic cancer risk; however, the results remain inconclusive (for review, see Ref. 2). Coffee consumption was initially reported to increase pancreatic cancer risk by a factor of

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2–3 (7), but subsequent studies have failed to reproduce this finding (2, 8). Some studies have reported an increased risk with high consumption of meat and low consumption of fruit, whereas low-to-moderate consumption of alcoholic beverages does not appear to be associated with an increased risk for pancreatic cancer (2).

Organochlorines are major environmental pollutants and include DDT[3] and PCB compounds. DDT was widely used as a pesticide, whereas PCBs are a group of chemically related synthetic compounds used for a variety of industrial and commercial purposes. Although DDT and PCBs were removed from the United States market in the 1970s, residual exposure continues because of the environmental persistence of these compounds. Exposure to DDT (and its metabolite DDE) and PCBs occurs in occupational and environmental settings, either directly or through food or other environmental sources. An increased risk for pancreatic cancer has been reported among individuals occupationally exposed to DDT and other organochlorine chemicals (for reviews, see Refs. 9 and 10). Elevated risks for pancreatic cancer were reported for DDT manufacturers (11), and to a lesser extent for workers potentially exposed to PCBs (12). Because organochlorines are lipophilic and resistant to further metabolism, serum levels of DDE and PCBs can be used as a surrogate measure of long-term exposure (13). To date, there has been only one report on the possible associations of organochlorine exposure with genetic alterations in pancreatic cancer (14).

Activating point mutations in codon 12 of K-*ras* are among the most common oncogene alterations in human adenocarcinomas, especially in pancreatic carcinoma (for reviews, see Refs. 15–17). In the earliest report, more than 90% of pancreatic carcinomas were shown to harbor K-*ras* codon 12 mutations (18), whereas more recent and larger studies have indicated a prevalence of about 75% (19, 20). K-ras is one of a family of three *ras* oncogenes that also includes the Harvey- and N-*ras* oncogenes. The *ras* oncogenes encode for closely related GTP-binding proteins that can acquire transforming potential when altered in one of the critical positions at codons 12, 13, or 61. Under normal circumstances, ras proteins are involved in growth signal transduction within the cell, similarly to "second messenger" G-proteins (for review, see Ref. 21). Cancer-associated point mutations occur almost exclusively in codon 12 of the K-*ras* oncogene.

Inactivation of the *p53* tumor suppressor gene is very common in almost all human cancers (for review, see Ref. 22). Normal p53 protein functions in cell cycle regulation, in maintenance of genomic stability, and in controlled cell death (apoptosis). A mutated p53 protein is capable of inactivating the normal function of p53 in the cell, even in the presence of the normal (wild-type) protein. Most inactivating mutations in *p53* consist of single-point mutations in evolutionarily conserved domains that change the amino acid composition of the resulting p53 protein. The majority of inactivating mutations in *p53* lead to an increased stability of the p53 protein. Under normal conditions, p53 protein levels in the cell nucleus are not detectable by standard protein immunohistochemistry, but in cells with mutated *p53,* the accumulation of p53 protein is easily detectable. Inactivation of the *p53* tumor suppressor gene is common in pancreatic carcinoma and is found in 50–70% of cases (23–25).

[3] The abbreviations used are: DDT, dichlorodiphenyltrichloroethane; DDE, dichlorodiphenyltrichloroethylene; PCB, polychlorinated biphenyl; LCM, laser capture microdissection; UCSF, University of California at San Francisco.

The molecular analysis of pancreatic cancer is complicated by its infiltrative growth and by strong desmoplastic and inflammatory responses from the host. Because of these features, it can be very difficult to obtain tumor cell preparations that are pure enough for molecular analyses. One approach to obtaining pure tumor DNA is to culture primary tumors as xenografts in nude mice, but this approach requires living cells and is difficult and time-consuming (25). A recent technical development, LCM, has improved the isolation of near-pure tumor cell populations (26). LCM has been successful in the isolation of DNA and RNA from a variety of tissue specimens (27). The small surface area of microdissection makes LCM much more precise than manual microdissection using a needle or surgical blade.

For the present investigation, we used a case-case approach to test the hypothesis that known risk factors for pancreatic carcinoma are associated with characteristic molecular defects in the tumors. This information may lead to a better understanding of the molecular pathways that are present in pancreatic carcinogenesis.

Materials and Methods

Study Population and Tissue Samples. This study was conducted as part of an ongoing case-control study of more than 550 cases of pancreatic cancer in the San Francisco Bay Area. Eligibility and methods of recruitment have been described in detail elsewhere (28). Briefly, eligible cases consisted of all patients between 21 and 85 years of age who were diagnosed as having cancer of the exocrine pancreas, and who were residents of the San Francisco Bay Area Counties of San Francisco, Alameda, Marin, Contra Costa, San Mateo and Santa Clara. For this substudy cases were diagnosed between May 1, 1995, and October 1, 1998. Patients had to be capable of having an interview in English at the time of enrollment. Cases were identified through the Northern California Cancer Center's rapid case ascertainment and through the pathology records of the UCSF teaching hospitals. All of the subjects provided informed consent before participating.

At the start of this substudy, information on a total of 321 eligible patients was available from the San Francisco Bay Area, whereas 53 patients were enrolled from the UCSF pathology records. A total of 91 patients had surgical tumor material available for study, of which 78 were from the Bay Area and 13 were from UCSF pathology records. In 18 of these patients, formalin-fixed paraffin-embedded tumor samples could not be obtained, mainly because of hospital refusal. For an additional six patients, the archival samples that were obtained contained insufficient tumor material for analysis, resulting in a total of 67 patients for whom all information and tumor material were available for study. An additional six specimens were excluded from the analysis because of histopathology other than pancreatic ductal carcinoma (three cystadenocarcinomas, two cystadenomas, one small cell tumor with neuroendocrine features). Thus, a total of 61 patients had sufficient material available for molecular analysis.

Pathological Review and Grading of Pancreas Tumors. The microscopic slides from all 61 cases were reviewed independently by a clinical pathologist (G. P. F.), without knowledge of the submitting diagnosis. Tumors were graded as outlined in the Armed Forces Institute of Pathology Atlas on Tumors of the Pancreas (29), including glandular differentiation, number of mitoses per 10 high power microscopic fields (×100), and nuclear atypia. The extent of mucin production by the tumor was not deemed to be easily evaluable and was not included

among the grading factors. Each of the three factors was assessed independently and scored from 1 to 3. The three scores were added to provide a total score that ranged from 3 to 9. Grade I carcinomas had scores of 3 or 4, Grade II had scores of 5–7, and Grade III had scores of 8 or 9. With tumors exhibiting variations in degree of differentiation, grading was based on the more poorly differentiated area if this area was 5 mm or larger. Tumors were also designated as Grade III if the entire tumor exhibited poorly differentiated architecture regardless of the total score, or if two of the grading factors were in the highest score category regardless of the score of the third factor. These modifications were used in four tumors that had a disparity between a low mitotic count and an otherwise poorly differentiated tumor and that had limited material available for review. Information on the origin and stage of the tumors was extracted from the original pathology reports. Of the 61 carcinomas included in this study, 59 were identified as ductal adenocarcinoma of the pancreas (3 were located in the tail of the pancreas), and 2 tumors were diagnosed as carcinoma of the ampulla of Vater. In all except 4 cases, sufficient staging information was available from the original pathology report. Staging classification was performed according to the most recent pancreatic cancer classification system by the American Joint Committee for Cancer (30).

LCM. LCM works by examination of a standard 5-μm section under the LCM microscope and by bringing a plastic cap in direct contact with the cells of interest. Activation of the LCM infra-red laser briefly melts the thermoplastic ethylene vinyl polymer on the cap surface, which causes cells to adhere to the cap for removal from the slide. The selection of optimum areas of tumor for microdissection was based on several factors. These included the absence of intermixed or immediately adjacent benign pancreatic acinar, ductal, or islet tissue, the absence of marked inflammation or hemorrhage, and the presence of glandular or solid tumor tissue that appeared to be representative of the tumor in the material available. The areas chosen for microdissection were outlined in ink on the glass slides and matched to the same area on consecutive weakly stained slides used for microdissection. Sections were cut at 5 μm. LCM was performed with a 30-μm laser beam, firing approximately 500 laser hits at an amplitude of 50 mW for 50 milliseconds. The polymer caps with the microdissected cells were then transferred to a 0.5-ml microcentrifuge tube containing 50 μl of DNA lysis buffer [10 mM TRIS (pH 8.0), 0.2% Tween 20, and 100 μg/ml proteinase K]. The tubes were then inverted and incubated for 18–24 h at 56°C. Before PCR, the proteinase was inactivated by incubation at 95°C for 10 min.

After LCM, the glass slides with the microdissected tissue were coverslipped and examined with the light microscope to determine the accuracy and effectiveness of the microdissection. In those cases in which the tumor was missed or was dissected along with benign tissue, the microdissection was repeated. This evaluation of the microdissected slides, and the consequent decision to repeat the dissection or not, was carried out independently and without knowledge of the molecular analysis of the original microdissected sample.

Analysis of K-*ras* Mutations. K-*ras* mutations were determined by a previously described method using a semi-nested PCR approach followed by mutation enrichment (31). DNA sequence analysis was performed to determine the precise nucleotide change in codon 12 of K-*ras* (32). Briefly, 5 μl of the DNA preparation, equivalent to one-tenth of the sample, was used for a first round of PCR amplification. PCR-I was carried out using primer A (5′-GAA AAT GAC TGA ATA TAA ACT TGT GGT AGT TGG ACC T-3′) and primer D (5′-TCA TGA AAA TGG TCA GAG AAA CC-3′) in a total volume of 25 μl for 35 cycles. To enrich for mutant K-*ras* codon 12 sequences, 10 μl of PCR-I was then used for digestion by the restriction endonuclease MvaI (Boehringer Mannheim, Indianapolis, IN). MvaI specifically cuts the wild-type K-*ras* sequence but not any sequences that are mutant at codon 12 of K-*ras*. For each sample, a second round of PCR amplification was then carried out using the PCR-I product (which was unenriched for mutant sequences), and in a separate tube, with the MvaI-digested PCR-I product (which was enriched for mutant sequences). PCR-II was performed with primers A and B (5′-TCA AAG AAT GGT CCT GGA CC-3′) in a total volume of 50 μl for 15 cycles. Other PCR parameters and conditions were as described previously (31, 32). The unenriched and mutant-enriched PCR-II products were then subjected to digestion with MvaI followed by agarose gel electrophoresis to distinguish between wild-type and codon 12 mutant K-*ras*. Samples were considered mutant when the unenriched PCR product showed a visible mutant signal that we estimated corresponded to a mutant contribution of at least 10% of the original LCM microdissected sample. To determine the specific nucleotide change at codon 12 of K-*ras*, mutant samples were subjected to automated DNA sequence analysis. For this purpose, the mutant-enriched PCR-II product was purified using QIAquick columns (Qiagen, Valencia, CA), used for DNA cycle sequencing with the ABI Prism dRhodamine terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, CA).

Immunohistochemistry for p53 Tumor Suppressor Protein. Immunohistochemical staining for p53 was carried out with monoclonal antibody Bp-53–11 using the NexES automated staining system (Ventana Medical Systems, Tucson, AZ) according to instructions by the manufacturer. Five-μm tissue sections were collected on positively charged slides, deparaffinized with xylene, and rehydrated in ethanol series. Antigen retrieval was performed by boiling the samples for 5 min in 0.1 M citrate buffer [0.1 M citric acid monohydrate and 0.1 M sodium citrate (pH 6.0)]. Final detection was performed with a standard biotin-avidin detection kit (Ventana Medical Systems). In the large majority of the cases, immunohistochemical staining for p53 was intense and localized in the nucleus of about 80–90% of the tumor cells. A minimum of 10% of the tumor cells had to demonstrate nuclear staining to be counted as p53 immunohistochemistry-positive.

Environmental Exposures. A detailed in-person interview was conducted in the subject's home or at a place convenient to the subject to obtain information on environmental exposures (diet, smoking history, and coffee consumption) and medical history along with demographic factors. No proxy interviews were conducted. Self-reported information on cigarette smoking was obtained for age of first smoking, number of years smoked, and number of cigarettes per day (for each period of smoking). Intervals that reflected a change in the smoking pattern were also assessed to obtain a more accurate measure of lifetime cigarette smoking. Coffee consumption in the year prior to diagnosis was obtained via a semiquantitative food frequency questionnaire. Cut points in the distribution of coffee consumption were selected based on the distribution in the sample.

Blood Sampling and Analysis. For patients enrolled between October 1, 1996, and October 1, 1998, nonfasting blood samples were obtained about 3 months postdiagnosis and were used for chemical analysis of organochlorines. These serum samples were analyzed for organochlorines as described previously

(33). Briefly, each sample was extracted by solid phase extraction and then analyzed on two separate gas chromatographs with electron capture detection. Samples were adjusted for recovery of the solid phase extraction method using the values reported in Ref. 33. Results in ng/ml serum were obtained for *p*,*p*′-DDE, *p*,*p*′-DDT, and 11 PCB congeners. Serum specimens were analyzed for cholesterol and triglycerides using enzymatic methods. Lipid-corrected organochlorine levels were created by dividing the recovery-adjusted estimates by the lipid content of the serum sample. The use of lipid-corrected values has been shown to account for differences in lipophilic chemical levels between fasting and nonfasting samples (13). The sum of all PCB congener values measured was used as a measure for total PCBs level. A detailed analysis of organochlorine levels in this patient population, including a discussion on possible effects of body size changes, has been published previously (28).

Statistical Analysis. Data analysis consisted of descriptive statistics using χ^2 test, χ^2 test for trend, and Fisher's exact test. Comparisons of normally distributed data, such as pack-years, were performed using Student's *t* test. Comparisons of non-normally distributed data, such as organochlorine levels, were performed using Wilcoxon's rank test. The statistical analyses were performed using SAS/STAT statistical software (SAS Institute, Cary, NC). All of the tests were two-sided and a value of $P < 0.05$ was considered statistically significant.

Results

Patient Characteristics and Selection. A total of 61 patients with pancreatic adenocarcinoma were included in this analysis. To assess a possible selection bias because of the limited number of cases with biopsy material available, a comparison of the main demographic, clinical, and exposure characteristics was made between patients enrolled through 1998 in the population-based case-control study of pancreatic cancer in the San Francisco Bay Area and the patients in this study (Table 1). None of these parameters varied significantly between the overall study and this subgroup, although cases with tumor material available were more likely to be female and to drink less coffee.

The clinical characteristics and the pathological findings of the 61 patients enrolled in this study are listed in Table 2. For each of these patients, neoplastic tissue was obtained and serial 5-μm sections were made from the formalin-fixed, paraffin-embedded tumor blocks. Serial sections were used for standard H&E staining, for immunohistochemistry, and for molecular analyses. Because surgical specimens of pancreatic cancer may only contain a minority of tumor cells, we used LCM to dissect the malignant cells away from stromal and inflammatory cells (26, 27).

LCM. An example of pancreatic cancer histology and the LCM procedure is shown in Fig. 1. A standard H&E-stained tumor section is shown in Fig. 1*A*, and the same tumor area is shown in Fig. 1*B* as seen with the LCM microscope. Ductal patterns are clearly visible in this field, although more subtle morphological features may be difficult to distinguish. The next two panels (Fig. 1, *C* and *D*) depict an example of the LCM slide after microdissection and the corresponding microdissected tumor material. In this study, capture was usually near complete, although in individual cases, a smaller fraction of the targeted cells was transferred to the cap. Examination of the caps after overnight incubation with DNA isolation buffer indicated complete digestion of cellular material (not shown). For each tumor sample, approximately 500 laser hits were performed. On average, about 5 cells are removed per laser hit, resulting in an estimated yield of 2500 cells per sample. The equivalent of 125 cells (which should contain 250 copies of the K-*ras* oncogene) was used for PCR amplification. To verify that there was sufficient amplifiable DNA present in these samples, K-*ras* PCR analysis was performed on 5-fold serial dilutions of representative tumor samples and were compared with dilutions of known amounts of placenta DNA. Fig. 2 shows 5-fold serial dilutions of 0.4 ng of placenta DNA (equivalent to about 250 gene copies), which gives a signal down to a dilution of about 1 gene copy, but not at lower dilution (*last 3 lanes* for placenta DNA). Microdissected DNA amplifies at an approximately equivalent dilution, indicating that each microliter of the LCM DNA preparation yields a sufficiently large number of gene copies to allow adequate tumor representation.

To determine the contribution of cells that were picked up by nonspecific adherence to the cap, we included controls for which the laser was not activated. In these controls, the cap was allowed to touch the tissue, but no laser hits were performed (*Cap only* lanes). The signal obtained in this way indicated the presence of a small fraction of cells that adhered to the caps but only in the undiluted specimens. These experiments demonstrated a high level of enrichment for cells in the laser field and only a minimal contamination from other cells. In addition to an

Table 1 Comparison of patient characteristics between the original participants in the ongoing population-based case-control study of pancreatic cancer and the subset with tumor blocks available

Characteristic	Ongoing case-control study $n = 321$	Subset with tumor blocks $n = 61$
Age at diagnosis (±SD)	64.9 (±10.7)	63.9 (±11.3)
Sex		
Female	161 (49.8%)	34 (55.7%)
Male	160 (50.2%)	27 (44.3%)
Race		
African-American	30 (9.3%)	4 (6.6%)
Asian	21 (6.5%)	4 (6.6%)
White	267 (83.2)	53 (86.8%)
Other	3 (1.0%)	0 (0%)
Diabetes[a]		
Yes	31 (9.7%)	8 (7.0%)
Average yr prior (±SD)	7.8 (±5.9)	8.3 (±6.1)
Range (yr)	2–22	2–21
Smoking[b]		
Ever	205 (63.9%)	37 (60.7%)
Never	115 (35.8%)	24 (39.3%)
Unknown	1 (0.3%)	0 (0%)
Coffee consumption[c]		
Less than 1 cup/day	149 (46.4%)	18 (29.5%)
1 or more cup/day	170 (53.0%)	43 (70.5%)
Unknown	2 (0.6%)	0 (0%)
Organochlorine levels[d]		
DDE (ng/g lipid)		
Mean (±SD)	2054 (±2038)	1848 (±1377)
Median	1287	1395
Range	187–16679	187–5352
PCBs (ng/g lipid)		
Mean (±SD)	433 (±412)	474 (±422)
Median	329	426
Range	ND[e]–2642	1–1840

[a] Self-reported diabetes more than 1 year before pancreatic cancer diagnosis.
[b] Defined as >100 cigarettes smoked in a lifetime.
[c] Total coffee consumption.
[d] Data from Hoppin *et al.* (28).
[e] ND, not detectable (detection limit for each PCB is 0.2 ng/ml).

Table 2 Characteristics of 61 patients with pancreatic carcinoma and univariate analysis of K-*ras* codon 12 mutation in pancreatic cancer patients, San Francisco Bay Area 1995–1998

Characteristic	*n*	K-*ras* negative *n* = 15	K-*ras* positive *n* = 46	*P*
Age at diagnosis (±SD)	61	64.6 (±9.8)	63.9 (±11.3)	0.84[a]
Sex				0.77[b]
Female	34	9 (60%)	25 (54%)	
Male	27	6 (40%)	21 (46%)	
Race				1.00[c]
African-American	4	1 (7%)	3 (7%)	
Asian	4	1 (7%)	3 (7%)	
White	53	13 (86%)	40 (86%)	
Diabetes[d]				0.002[b]
Yes	8	6 (40%)	2 (4%)	
Average yr prior (±SD)		10.8 (±6.5)	4 (±2.8)	
Range (yr)		4–21	2–4	
Tumor stage				0.08[e]
I	13	6 (40%)	7 (15%)	
II	14	3 (20%)	11 (24%)	
III	25	4 (26%)	21 (45%)	
IV	5	1 (7%)	4 (9%)	
Unknown	4	1 (7%)	3 (7%)	
Glandular differentiation				0.59[e]
Well	28	6 (40%)	22 (48%)	
Moderately	15	7 (47%)	8 (17%)	
Poorly	18	2 (13%)	16 (35%)	
Nuclear atypia				0.32[e]
Mild	15	5 (33%)	10 (22%)	
Moderate	20	5 (33%)	15 (33%)	
Severe	26	5 (33%)	21 (45%)	
Mitotic index[f]				0.71[e]
Low	36	9 (60%)	27 (59%)	
Medium	18	5 (33%)	13 (28%)	
High	7	1 (7%)	6 (13%)	
Tumor grade				0.54[e]
I	23	6 (40%)	17 (37%)	
II	16	5 (33%)	11 (24%)	
III	22	4 (27%)	18 (39%)	

[a] Student's *t*-test.
[b] Fisher's exact test.
[c] χ^2 test.
[d] Self-reported diabetes more than 1 year prior to pancreatic cancer diagnosis.
[e] χ^2 test for trend.
[f] Mitotic figures per 10 high-power fields: low, 0–5; medium, 6–10; high, >10.

analysis on LCM enrichment, the following negative controls were included: lysis buffer only, and water-only in PCR-I and PCR-II amplifications.

K-*ras* Codon 12 Mutational Analysis. An example of the initial screen for K-*ras* codon 12 point mutations is shown in Fig. 2*B*. Aliquots of both the unenriched and mutant-enriched PCR-II product are digested with MvaI to yield a wild-type K-*ras* DNA fragment of 111 bp and a mutant fragment of 147 bp. The original wild-type:mutant ratio is represented in the unenriched lanes, whereas an almost pure mutant DNA population can often be achieved in the mutant-enriched lanes. The latter property makes these PCR preparations amenable for automated sequence analysis (32). Fig. 3 demonstrates DNA sequence analysis of four different tumors. Of the total of 61 pancreatic cancers, 46 (75%) had a mutant K-*ras* codon 12 sequence, whereas 15 (25%) of the tumors had the normal "GGT" sequence at codon 12. The distribution of activating mutations was: 25 (54%) "GAT," 14 (30%) "GTT," 6 (13%) "CGT," and 1 (2%) "TGT."

A comparison between the characteristics of all 61 patients and the results of the K-*ras* codon 12 mutational analysis is shown in Table 2. There was no significant difference with respect to age-at-diagnosis, sex, or race. Patients with a self-reported history of diabetes more than 1 year prior to cancer diagnosis had a significantly lower frequency of K-*ras* mutations than patients who did not have this diagnosis (Fisher's exact test: $P = 0.002$). The mean time between diagnosis of diabetes and pancreatic cancer diagnosis was 9.1 years (range, 2–21 years) for all patients with a diagnosis of diabetes more than 1 year prior to the pancreatic cancer diagnosis.

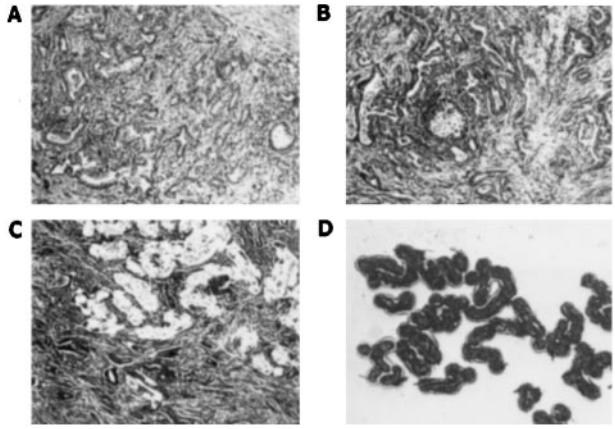

Fig. 1. Example of histopathology and LCM of pancreatic cancer. *A*, H&E-stained tumor section (×25) with stromal and inflammatory cells surrounding the tumor ducts; *B*, the same tumor area under the LCM microscope (×40); because of the absence of a coverslip, larger architectural features—but not finer cellular details—are visible; *C*, tumor section after microdissection (×40), illustrating precise microdissection of tumor ducts without inclusion of adjacent reactive tissue; *D*, microdissected tumor cells adherent to the LCM cap (×40) and subsequently used for DNA isolation.

The following histopathological features of the tumors were determined: glandular differentiation, nuclear atypia, and mitotic index. The combination of these three factors resulted in a score for tumor grade (I through III). In addition, staging information was obtained from the pathology records. K-*ras* mutational status was not associated with any of these factors, although there was some evidence for a higher incidence of K-*ras* mutations with increasing tumor stage ($P = 0.08$, χ^2 test for trend).

The analysis of the relationship between environmental exposures and K-*ras* codon 12 point mutations is shown in Table 3. There was no significant difference between K-*ras* positive and negative tumors with respect to smoking history for the following comparisons: ever *versus* never smoker, non-smoker *versus* current smoker, average pack-years, or duration of smoking. Coffee consumption was analyzed for caffeinated, decaffeinated, and total coffee consumption. No significant differences were found with respect to K-*ras* mutation and coffee consumption. Patients with K-*ras* positive tumors tended to have lower serum levels of DDE, but this difference did not reach statistical significance. Median serum DDE levels were 1951 ng/g lipid *versus* 1287 ng/g lipid in patients with K-*ras* negative and K-*ras* positive tumors, respectively ($P = 0.16$, Wilcoxon's test). There was no difference between the K-*ras* negative and positive groups with respect to median levels of total PCBs ($P = 0.34$, Wilcoxon's test). With the present sample size, the minimum detectable difference was 1758 ng/g lipid for DDE (*versus* 664 ng/g lipid observed) and 285 ng/g lipid for PCBs (*versus* 18 ng/g lipid observed), at 80% power and with a 0.05 significance level.

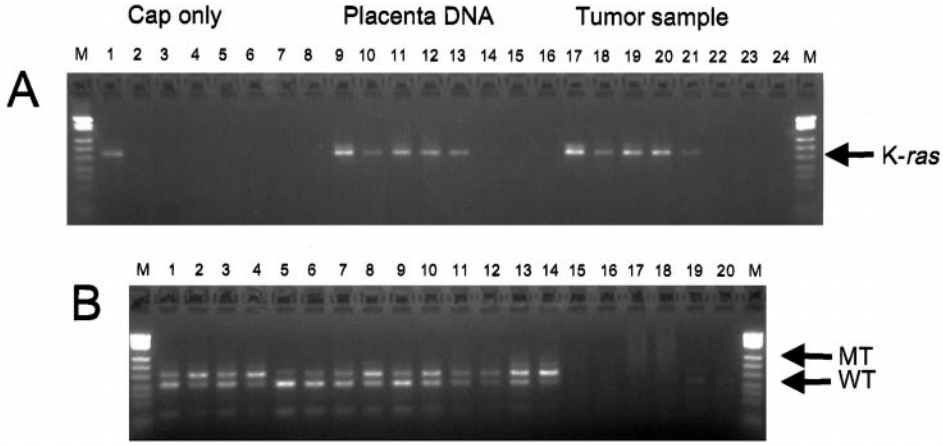

Fig. 2. Analysis of pancreatic cancer samples by mutant enriched PCR for K-*ras*. In *A*, as shown in the middle part of the gel (*Lanes 9–16*), placenta DNA was diluted from 0.4 ng (~250 gene copies) in 5-fold steps down to less than 1 copy per reaction. *On the right* (*Lanes 17–24*), 5-fold dilutions of a representative tumor sample starting from 5 μl (1/20 of the LCM preparation) per reaction are shown. In the *left part of the gel* (*Lanes 1–8*), 5-fold serial dilutions of one of several LCM preparations from caps that only touched the tissue without any activation of the laser are shown. The assay can detect as low as 1 gene copy using 35 + 15 PCR cycles. In *B*, the equivalent of approximately 250 gene copies (1/20 of the LCM preparation) was amplified from seven microdissected pancreas cancer cases and digested with MvaI. The *top band*, the undigested mutant K-*ras* sequence (147 bp); the *lower band*, the digested wild-type K-*ras* band (111 bp). The *left lane* (*odd numbers*) for each sample is unenriched for mutant K-*ras* and, therefore, corresponds to the original wild-type:mutant ratio in the tumor, whereas the *right lane* (*even numbers*) was enriched for mutants at codon 12 of K-*ras*. The sample in *Lanes 5* and *6* was wild-type for K-*ras*, whereas all other samples had mutant signals that enriched after MvaI digestion. *Lanes 15* and *16*, *17* and *18*, and *19* and *20* are cap only and PCR-I water and PCR-II water only controls, respectively. *M* lanes, loaded with *Msp*I-digested pUC18 as a size marker.

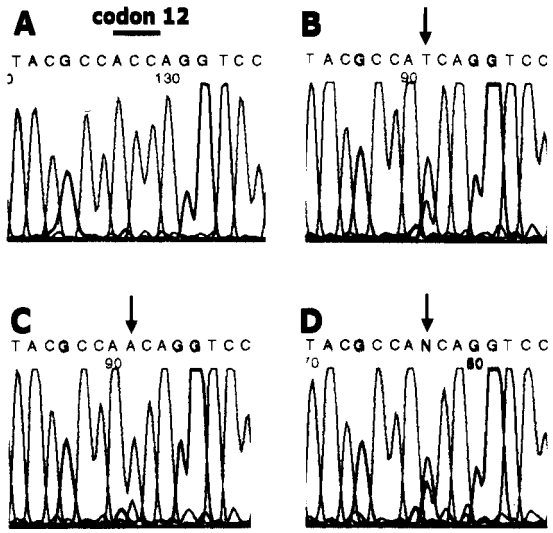

Fig. 3. Automated DNA sequence analysis of K-*ras* codon 12 mutations in pancreatic cancer. The cDNA sequence was used for this analysis. Hence, the wild-type K-*ras* codon 12 sequence reads "ACC," corresponding to the GGT sequence in the coding strand. *A*, example of wild-type (GGT) K-*ras* at codon 12; *B*, example of mutant K-*ras* (GAT); *C*, example of mutant K-*ras* (GTT); *D*, example of mutant K-*ras* (GAT) with a lower level of enrichment for the mutant sequence. *Arrows*, position 2 of the K-*ras* codon 12 sequence.

Table 3 Environmental exposures and K-*ras* codon 12 mutation in pancreatic cancer patients, San Francisco Bay Area 1995–1998

Characteristic	*n*	K-*ras* negative *n* = 15	K-*ras* positive *n* = 46	*P*
Smoking history				N.S.[a]
Never smoker	24	6 (40%)	18 (39%)	
Ever smoker	37	9 (60%)	28 (61%)	
Pack-year (±SD)		50.9 (±48.2)	31.0 (±20.6)	
Years of smoking (±SD)		38.3 (±15.4)	32.6 (±12.1)	
Current smokers	13	3	10	
Coffee consumption	61			
Caffeinated				1.00[b]
<1 cup/day		7 (47%)	17 (37%)	
≥1 cups/day		8 (53%)	29 (63%)	
Decaffeinated				0.43[b]
<1 cup/day		14 (93%)	38 (83%)	
≥1 cups/day		1 (7%)	8 (17%)	
Total coffee				1.00[b]
<1 cup/day		7 (47%)	11 (24%)	
≥1 cups/day		8 (53%)	35 (76%)	
Organochlorine levels	24	7	17	
DDE (ng/g lipid)				0.16[c]
Mean (±SD)		2579 (±1648)	1547 (±1203)	
Median		1951	1287	
Range		860–5347	187–5352	
PCBs (ng/g lipid)				0.34[c]
Mean (±SD)		633 (±594)	408 (±337)	
Median		304	322	
Range		180–1840	1–1306	

[a] N.S. Not significant; analyses done for: ever *versus* never smoker ($P = 1.00$, Fisher's exact test); nonsmoker *versus* current smoker ($P = 1.00$, Fisher's exact test); pack-year ($P = 0.26$, Student *t*-test); duration of smoking ($P = 0.22$, Student's *t*-test).
[b] Fisher's exact test.
[c] Wilcoxon's rank-sum test.

p53 Immunohistochemical Analysis. A total of 49 patients had sufficient tumor material available for immunohistochemical analysis of p53 protein accumulation. An example of such an analysis is shown in Fig. 4. Staining for p53 was always limited to the nucleus of histologically abnormal cells. A comparison of patient characteristics and p53 immunohistochemical results is shown in Table 4. There was no statistically significant difference between the p53-negative and positive groups with respect to age-at-diagnosis, sex, race, or prior history of diabetes. A strong association between p53 immunohistochemical staining and glandular differentiation ($P = 0.002$, χ^2 test for trend) and nuclear atypia ($P = 0.0007$, χ^2 test for trend) was

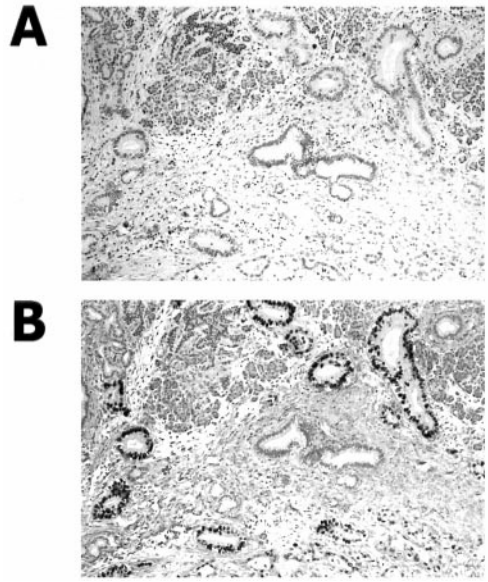

Fig. 4. Immunohistochemistry of p53. *A*, negative control, in the absence of a specific antibody for immunohistochemical staining (×25). *B*, a consecutively cut tissue section stained for the p53 tumor suppressor protein (×25). There is intense nuclear staining in the pancreatic tumor ducts and the absence of staining in a large atypical, but nonmalignant, pancreatic duct in the middle of the figure.

observed, but p53 staining was not associated with mitotic activity of the tumors. The composite of these three parameters, tumor grade, was also associated with p53 immunohistochemical staining ($P = 0.0038$, χ^2 test for trend). There was an indication of heterogeneity in p53 staining by tumor stage ($P = 0.01$, Fisher's exact test); however, there was no evidence of a trend toward higher stage ($P = 0.88$, χ^2 test for trend).

A relationship between p53 staining and smoking history was analyzed by comparison of ever- *versus* never smoking, current smoking *versus* prior- and never-smoking, mean pack-years, and mean duration of smoking. Eleven of 18 never smokers *versus* 14 of 31 ever smokers were positive for p53 staining, whereas ever smokers had an average of 36 and 39 pack-years in the p53 positive and negative groups respectively. None of these comparisons showed statistically significant differences. There was no difference with respect to coffee consumption (caffeinated, decaffeinated, or total) in relation to p53 immunohistochemical staining. In the p53 positive group, 11 patients had an average total coffee consumption of <1 cup per day, whereas 14 patients drank an average of ≥ 1 cups per day. In the p53 negative group, these numbers were 14 and 10, respectively ($P = 0.396$, Fisher's exact test). Median serum levels of DDE and PCBs were not significantly different between the p53 negative and positive groups. DDE levels were 1618 *versus* 1993 ng/g lipid and PCBs were 420 *versus* 491 ng/g lipid in the p53 positive ($n = 12$) and negative ($n = 10$) groups, respectively.

There was some evidence of an association between K-*ras* codon 12 mutations and p53 staining. Twenty-two tumors were mutant for both K-*ras* and p53, 9 were wild-type for both, 15 tumors were K-*ras* wild-type but p53 mutant, and 3 tumors were K-*ras* mutant but p53 wild-type ($P = 0.051$, Fisher's exact test).

Table 4 Characteristics of 49 patients with pancreatic carcinoma and univariate analysis of p53 immunohistochemical staining in pancreatic cancer patients, San Francisco Bay Area 1995–1998

Characteristic	*n*	p53 negative *n* = 24	p53 positive *n* = 25	*P*
Age at diagnosis (±SD)	49	65.6 (±10.8)	63.7 (10.8)	0.76[a]
Sex				0.25[b]
Male	24	14 (58%)	10 (40%)	
Female	25	10 (42%)	15 (60%)	
Race				0.11[c]
White	42	22 (92%)	20 (80%)	
African-American	4	1 (4%)	3 (12%)	
Asian	3	1 (4%)	2 (8%)	
Diabetes[d]				0.46[b]
Yes	8	5 (21%)	3 (12%)	
No	41	19 (79%)	22 (88%)	
Tumor stage				0.01[c]
I	11	8 (33%)	3 (12%)	0.88[e]
II	10	3 (13%)	7 (28%)	
III	20	7 (29%)	13 (52%)	
IV	5	5 (21%)	0 (0%)	
Unknown	3	1 (4%)	2 (8%)	
Glandular differentiation				0.002[e]
Well	23	15 (63%)	8 (32%)	
Moderately	13	8 (33%)	5 (20%)	
Poorly	13	1 (4%)	12 (48%)	
Nuclear atypia				0.0007[e]
Mild	13	10 (42%)	3 (12%)	
Moderate	17	11 (46%)	6 (24%)	
Severe	19	3 (13%)	16 (64%)	
Mitotic index[f]				0.17[e]
Low	29	16 (67%)	13 (52%)	
Medium	15	7 (29%)	8 (32%)	
High	5	1 (4%)	4 (16%)	
Tumor grade				0.004[e]
I	19	12 (50%)	7 (28%)	
II	13	10 (42%)	3 (12%)	
III	17	2 (8%)	15 (60%)	

[a] Student's *t*-test.
[b] Fisher's exact test.
[c] χ^2 test.
[d] Self-reported diabetes diagnosed >1 year prior to pancreatic cancer diagnosis.
[e] χ^2 test for trend.
[f] Mitotic figures per 10 high-power fields: low, 0–5; medium, 6–10; high, >10.

Discussion

Pancreatic cancer is a highly fatal cancer with few identified risk factors. Hospital-based studies have demonstrated the importance of several genetic alterations in cancer-associated genes that are common in pancreatic cancer. These studies have been complicated by difficulties in obtaining pure tumor samples from archival tissue blocks, and they have not related genetic findings with known risk factors for pancreatic cancer.

K-*ras* and p53 Alterations. Mutations in codon 12 of the K-*ras* oncogene are very frequent in pancreatic cancer. Previous reports have indicated that between 70 and 90% of the tumors harbor such mutations (18, 19). In this study, 75% of the tumors harbored K-*ras* codon 12 mutations. The majority of these mutations were attributable to replacement of the normal GGT sequence by either GAT or GTT (replacing glycine with either aspartic acid or valine). The distribution of the different mutations in codon 12 of K-*ras* is remarkably similar to that reported in a pooled analysis of 13 separate studies (17).

Immunohistochemical staining patterns for p53 were found in about 50% of the pancreas cancers in this study, which is in agreement with previously reported frequencies (19, 25,

34). In general, p53 staining is associated with inactivation of the gene as determined by DNA sequence analysis, but differences between the two methods exist. Analysis by immunohistochemistry has the advantage that all of the mutations that result in a stabilized protein can be detected, even if they occur outside exons 5 through 8 to which most sequencing efforts are limited. Previous studies have shown that K-*ras* codon 12 mutations and *p53* tumor suppressor gene alterations occur independently of each other (34). Our data are in general agreement with this, although there was some evidence of an association between K-*ras* mutations and p53 accumulation. In this study, mutations in codon 12 of K-*ras* or p53 staining did not cluster in any particular patient group: associations with age-at-diagnosis, sex, or race have not been reported previously and are absent in this study as well.

K-*ras* and Diabetes Mellitus. One striking observation was the relative absence of K-*ras* mutations in pancreatic tumors from patients with a self-reported diagnosis of diabetes mellitus more than 1 year prior to the diagnosis of pancreatic cancer. The two patients who had K-*ras* mutation positive tumors reported diabetes 2 and 4 years prior to their pancreatic cancer diagnosis, respectively, whereas the six K-*ras* mutation negative cases had a minimum of 4 years between the reported onset of diabetes and the pancreatic cancer diagnosis. Our assessment of diabetes was based on a self-reported diagnosis, which was previously shown to be in good agreement with information obtained from medical records of pancreatic cancer patients (35). The first question regarding diabetes was: "Were you ever diagnosed as having diabetes, or sugar diabetes that lasted for one year or longer?," and was followed up with 9 additional questions regarding the specifics of the diagnosis. Given that all patients were asked the same question, all were pancreatic cancer cases, and none were aware of the K-*ras* status of the cancer, we do not expect any classification bias.

It is interesting to note that diabetes mellitus is a well-established risk factor for pancreatic cancer. A meta-analysis of 20 case-control and cohort studies demonstrated a relative risk of about 2 for patients with diabetes mellitus (4). In our study, pancreatitis is unlikely to be a confounder because none of the diabetic patients had a history of chronic or acute pancreatitis. The biology underlying an association of diabetes with pancreas cancer remains poorly understood. A possible factor might be the exposure of the pancreas to the growth-promoting effects of high levels of insulin in certain cases (36).

The close association of diabetes with a wild-type K-*ras* result is suggestive of a distinct tumorigenesis pathway in these patients. Indeed, evidence for separate tumor pathways has been proposed for pancreatic cancer in a recent report by Goggins *et al.* (37). A small minority of tumors exhibit microsatellite instability (RER phenotype) which is caused by defects in DNA mismatch repair capacity. The RER phenotype tumors were associated with the absence of K-*ras* codon 12 mutations, poor histological differentiation, a syncytial growth pattern in which cell borders are not well defined, and a better prognosis. Of the eight pancreatic cancers from patients who had a previous diagnosis of diabetes in this study, only one appeared to fit the criteria for the RER phenotype. It can, thus, be hypothesized that pancreatic cancers arising in patients with a long-standing history of diabetes may have a different genetic profile than those that are not associated with diabetes, and that these tumors are different from those with the RER phenotype. It would be of interest to determine whether such differences are reflected in tumor behavior and, eventually, patient outcome. Additional studies will be needed to study this specific question. Additional tumor characteristics (differentiation, mitotic index, nuclear atypia, and grade) did not appear to differ between those from diabetic patients or from non-diabetic patients, although the numbers for these comparisons are small.

Tumor Characteristics. Associations between K-*ras* mutational status and tumor grade and stage have generally been absent in pancreatic cancer (13, 17), although we did find a weak trend toward a higher frequency of K-*ras* mutations with increasing stage. Because our study consisted only of surgical cases, we must assume a selection for lower tumor stage, which may limit generalizability to more advanced cancers. However, the frequency and pattern of K-*ras* mutations observed in this study are consistent with previously reported series. None of the tumor grade criteria (glandular differentiation, nuclear atypia, and mitotic index) or the combined measure of these parameters were statistically different between the K-*ras* positive and negative groups. These findings are in agreement with results from previous studies (19, 37, 38).

When p53 staining was considered, a completely different picture in relation to histopathological features emerged. There were significant trends between increasing p53 positivity and both decreasing glandular differentiation and increasing nuclear atypia. Several studies on pancreatic cancer have shown a relationship between *p53* mutations and nuclear aneuploidy and poor differentiation (25, 38). A possible explanation for the association between p53 inactivation and nuclear abnormalities is the proposed role of the p53 tumor suppressor protein in the maintenance of genomic integrity (39). According to this scenario, inactivation of p53 leads to increased accumulation of additional chromosomal abnormalities, including those that lead to deletion of the *p53* locus itself (40). Accumulation of p53 protein was also more frequently found in higher stage tumors, although none of the five stage IV tumors in this study stained for p53. Some reports have indicated a similar association of p53 positivity with higher-stage tumors, and there are indications that *p53* inactivation may be associated with poor survival in pancreatic cancer (25, 38). Staining for p53 was not different between patients with or without a previous diagnosis of diabetes or for the other patient characteristics (Table 4).

Environmental Exposures. Although the occurrence of K-*ras* mutations is linked to smoking in tumor types such as lung cancer (41), such a relationship appears to be absent in this study. One previous report indicated a difference in K-*ras* mutation prevalence between ever- and never-smokers in pancreatic cancer, but there was no association with the number of pack-years smoked (19). Other studies have failed to show an association with smoking (25). In light of the modest increased risk of pancreatic cancer with smoking, the effect on K-*ras* mutational pattern is probably small.

An association between coffee drinking and pancreatic cancer was proposed in some early studies, but nearly all subsequent studies have failed to document such an increased risk (reviewed in Ref. 2). Recently, it was reported that coffee consumption was significantly higher in patients with K-*ras* positive tumors than in patients with K-*ras* negative tumors (42). However, our study did not find any clear relation between K-*ras* mutational pattern or p53 staining and coffee drinking. This was true for caffeinated coffee, decaffeinated coffee and total coffee drinking habits. Our data do not suggest an influence of coffee consumption on the pattern of K-*ras* mutations or *p53* inactivation in pancreatic cancer. However, it may be a limitation that our questionnaire asked about average total coffee consumption habits during the year prior to diag-

nosis, which may not capture the etiologically relevant exposure period.

Information linking organochlorine exposure and cancer is largely based on animal research and less so on epidemiological studies in humans (9, 10). In rodents, DDT and DDE have a possible mutagenic effect, whereas PCBs can act as tumor promoters. The United States Environmental Protection Agency has classified both DDT and PCBs as probable human carcinogens (43, 44). Occupational exposure to technical grade DDT was associated with an increased risk for pancreatic cancer in one study (11), whereas other studies have been too small to detect effects on specific cancer types (10). We did not find an association between serum DDE levels and pancreatic cancer risk in the larger case-control study from which the specimens reported here were obtained (28), whereas a different case-control study reported elevated organochlorines among pancreatic cancer cases (14). The limitations found with DDE apply to studies of occupational exposures to PCBs as well: only one of eight cohorts has demonstrated an elevated risk for pancreatic cancer (10, 12), although we observed a significant dose-response relationship for total PCBs in the larger case-control study (28). Our data suggest a possible link between DDE exposure and the absence of K-*ras* mutations ($P = 0.16$, Wilcoxon's test). This result would argue against a role of DDT as a mutagen acting in the K-*ras* pathway of pancreatic cancer tumorigenesis. There was also no difference in PCB levels between the K-*ras* wild-type and mutant groups. Although serum organochlorine levels were available only for a limited subset of patients, which limited the power of our comparisons, the measurements in this study were comparable with those found in the larger ongoing case-control study from which our specimens were derived (see Table 1).

LCM. LCM is a powerful tool for targeting specific cell populations (45). This is especially important for the molecular analysis of pancreatic cancer cells, among which small nests of tumor cells are frequently surrounded by abundant nonneoplastic reactive tissue. With only minor purification steps, LCM preparations yield DNA that is highly enriched for tumor and is of sufficient quality for multiple PCR analyses. This enrichment is particularly important for molecular analyses that require pure tumor cell populations such as analyses for sequence changes or for loss of heterozygosity. However, in some cases, it was difficult to locate the cells of interest under the LCM view, especially in the absence of larger architectural structures in the section. It was also clear that a fraction of the tissue adheres nonspecifically to the cap, even when the laser was not activated to melt the polymer. These cells were not expected to contribute significantly to the final DNA preparation, however, and this was shown in several experiments using serial dilutions in the K-*ras* PCR. These experiments also showed that the DNA preparations contained sufficient numbers of gene copies to be representative of the tumor. A minimum of 250 K-*ras* gene copies were calculated to be present at the start of each PCR assay, which should give sufficient representation of any abnormal sequences present in the tumor. Representation is an important aspect when using formalin-fixed tissue, which may degrade DNA and increase the risk of false-positive results (46).

In conclusion, we have assessed K-*ras* oncogene mutations and p53 protein accumulation in a population-based sample of pancreatic cancers to investigate the hypothesis that these genetic alterations may be associated with known or postulated risk factors for pancreatic cancer. One of the main findings was that activation of the K-*ras* oncogene, the most commonly mutated oncogene in pancreatic cancer, was rare in tumors obtained from patients with a history of diabetes. Additional studies will be needed to determine whether this reflects a separate pathway of tumorigenesis in these patients. Although previous reports have identified increased risk for pancreas cancer associated with cigarette smoking, possibly coffee consumption, and organochlorine levels in serum, these factors were not associated with either K-*ras* or p53 accumulation in the present study. In addition, strong evidence was found for a relationship between p53 accumulation and nuclear abnormalities, consistent with a role of p53 in the maintenance of genomic integrity.

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